5-8-2020

Novel method to determine paleodiet of extinct equid Merychippus sp. using dental calculus

Ranjit Virk

Novel method to determine paleodiet of extinct equid *Merychippus* sp. using dental calculus

An Honors College Project Presented to

the Faculty of the Undergraduate

College of Science and Mathematics

James Madison University

by Ranjit Zorawar Singh Virk

May 2020

Accepted by the faculty of the Department of Biology, James Madison University, in partial fulfillment of the requirements for the Honors College.

FACULTY COMMITTEE:

Project Advisor: Katrina E. Gobetz, Ph.D.
Associate Professor, Department of Biology

Reader: Corey L. Cleland, Ph.D.
Associate Professor, Department of Biology

Reader: Michael H. Renfroe, Ph.D.
Professor, Department of Biology

HONORS COLLEGE APPROVAL:

Bradley R. Newcomer, Ph.D.,
Dean, Honors College

PUBLIC PRESENTATION

This work is accepted for presentation, in part or in full, at N/A on N/A.

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Acknowledgements

I cannot express enough appreciation to my reading committee members, Dr. Corey L. Cleland and Dr. Michael H. Renfroe, for their contribution in the review and ensuring the successful completion of the thesis. Additionally, I would like to extend my thanks for the support from Dr. Kristopher E. Kubow and the James Madison Biology Department's Light Microscopy and Imaging Facility in the facilitation of the microscopy that was necessary for the data collection of this project. I would also like to thank the Smithsonian Museum of Natural History and the Deputy Collections Manager, Ms. Amanda Millhouse, for facilitating the acquisition of the *Merychippus* calculus samples that allowed this thesis project to go forward. Lastly, my gratitude extends to my research advisor, Dr. Katrina E. Gobetz, for all her guidance in the execution and writing of this thesis. Without the contribution of these individuals and institutions, the completion of this thesis would not have been accomplished.

Abstract

Within the Miocene Epoch, the emergence of grasslands within North America coincided with the incidence of higher-crowned teeth (hypsodonty) within the proto-horse *Merychippus* that allowed for the inclusion of these expanding grasslands as a food source. As herbivorous paleofauna consume plant matter, microscopic remains become incorporated within dental calculus and, due to their diagnostic morphology, can be used to identify dietary components. In *Merychippus*, the recovery of plant microfossils holds the potential to provide greater clarity on the paleodiet of these equids. In the present study, the purpose was to quantify and compare *Merychippus* paleodiet constituents among sample groups. Calculus samples obtained from four isolated individual *Merychippus* teeth contained plant microfossils that reflect dietary elements. Microfossil assemblages from each *Merychippus* specimen were identified to the categories of definite and possible hardwood, indeterminate grass, pooid grass, sedge, tracheid and plant fiber microfossils based upon morphological characteristics. Abundant in all assemblages were microfossils derived from hardwood plant species in the form of leaf epidermal aggregate pieces and vessel elements, indicating a possibly significant dietary component. In addition, sedge phytoliths present in three assemblages and pooid phytoliths present in all assemblages reflect the inclusion of plants within Family Cyperaceae and the Poaceae Subfamily Pooideae, respectively. The combined presence of hardwood leaf epidermal aggregate, vessel elements, and pooid grass phytoliths within all fossil assemblage groups indicated a mixed browse/grass diet that included grass of definite C_3 photosynthetic character for all *Merychippus* specimens. Within a broader context, this study generalizes aspects of the *Merychippus* paleoenvironment, and stands as an evaluation for the sole use of microfossils derived from dental calculus as a means for diet determination.

Introduction

The early Miocene Epoch (23.0-5.3 MYA) is marked by the expansion of grasslands within the formerly wooded ecosystems of North America, which created a new ecological niche for herbivores of this time period (Macfadden, 2000; Wang et al., 1994; Webb, 1977). Coinciding with the proliferation of North American grasslands was the emergence of higher-crowned teeth (hypsodonty) in numerous herbivorous clades that allowed for more effective utilization of grass as a food source (Macfadden, 2000; MacFadden, 1997; MacFadden et al., 1994; Pérez-Crespo et al., 2016). The high silica content of grasses and the buildup of grit on leaf surfaces associated with feeding near the ground caused increased erosion of tooth enamel. Hypsodonts offset the accelerated wear from these abrasive substances by carrying the adaptive advantage of more resistant, higher-crowned teeth (Janis, 1988; MacFadden et al., 1999; Wang et al., 1994). Among the herbivorous clades that developed hypsodonty during the early Miocene, perhaps some of the most profoundly affected by this transition were members of the Family Equidae. Equids experienced extensive adaptive radiation during the opening of the North American plains, ultimately resulting in the co-existence of 13 genera by the late Miocene (Macfadden, 2000). The rapid evolutionary expansion of paleo-equid species during the Miocene, running parallel with extensive morphological changes in tooth crown length, stands as evidence to support the environmental transition from woodland to expanding grasslands in North America (Wang et al., 1994; Webb, 1977).

Another facet of the adaptive radiation of North American paleo-equids is that the proto-horse genus that first featured the modern equid trait of hypsodonty is considered to be the first to utilize the newly expanded grasslands as a primary food source. It is also the standard theoretical ancestor for hypsodont Mio-Pliocene horses leading to modern *Equus* (Evander,

1986). This equid from, which the prolific adaptive radiation of *Equidae* in the Miocene stems, is *Merychippus* (MacFadden et al., 1991; MacFadden and Hulbert, 1988; Stebbins, 1981). The proto-horse *Merychippus* was a tridactyl savannah-dwelling herbivore with an estimated body mass of between 71.0-100.6 kg and an estimated height ranging from 0.8 to 1.3 m or 10 hands, roughly Shetland pony sized (Fig. 1) (Macfadden, 1986; Thomason, 1986). Enhancing the importance of this genus further is, analysis on the diet of *Merychippus* species can provide greater clarity regarding floral composition of North American grasslands during their expansion in the early Miocene. The modern analogues considered to most closely resemble the early Miocene North American grasslands, but not in every respect, are the modern plains of North America and the savanna grasslands of Africa. Both biomes feature predominantly grass species that carry out photosynthesis via the C_4 photosynthetic pathway (Wang et al., 1994). Based upon modern analogues and the appearance of hypsodonty in the fossil record, the diet of North American herbivorous fauna of the early Miocene is believed to comprise principally C_4 grasses (MacFadden, 1997; Wang et al., 1994). However, studies utilizing carbon isotopic analyses to construct paleodiets of extinct herbivores provide evidence that supports an alternative diet composition.

Analysis of carbon isotopic ratios from within tooth enamel provides a means to determine the relative ratio of plants utilizing C_3 or C_4 photosynthetic pathways in the diet of herbivorous paleofauna (MacFadden et al., 1999). Plants utilizing a C_3 pathway include shrubs, trees, woody plants, leafy plants, and grasses that grow in xeric and/or cool, seasonal conditions. In contrast, plants that perform C_4 photosynthesis include grasses from temperate or tropical regions that are less resistant to seasonally cooler temperatures. Because C_3 and C_4 pathways produce different ratios of C^{12} and C^{13} stable carbon isotopes within their cells, the analysis of

these ratios provides a reliable gauge of whether the plants an organism ate were C_3 or C_4 photosynthesizers (MacFadden et al., 1999; Wang et al., 1994).

Regarding *Merychippus*, paleodiet analyses of carbon isotopes indicate that species in this genus consumed a diet of predominantly C_3 plants or an unbalanced mixed diet of C_3/C_4 plants with a heavier incidence of C_3 plants (Pérez-Crespo et al., 2016; Wang et al., 1994). Moreover, separate analyses conducted on the wear patterns of *Merychippus* teeth corroborate isotopic data to suggest that *Merychippus* species consumed a combination of grasses and browse, with the distinction that the photosynthetic behavior of the grasses cannot be elucidated through wear pattern data alone (Hayek et al., 1992). Analogous studies of contemporary herbivorous paleofauna corroborate results of *Merychippus* paleodiet studies, providing evidence that the predominant dietary component of North American herbivores during this time period was C_3 plants (Macfadden, 2000; Wang et al., 1994). A C_3 plant-dominated diet suggests that a floral depiction of the expanding North American grassland biome is sparse in C_4 grasses, and therefore holds no resemblance to any modern analogue (Cerling et al., 1997; Wang et al., 1994). However, an emerging method of herbivorous paleodiet determination, the analyses of plant microremains in dental plaque deposits, holds the potential to provide more precise discrimination of the plants that *Merychippus* sp. included within their diet than data obtained from tooth wear patterns or carbon isotopic ratios (Asevedo et al., 2012; Gobetz and Green, 2004).

Determining diet using fossilized plant material trapped in plaque is a relatively new method in vertebrate paleontology, even though the method has been broadly applied in paleoanthropology (Asevedo et al., 2012; Henry and Piperno, 2008). As an animal consumes plant leaves, stems, and twigs, microscopic remnants of chewed plant material become trapped

within the dental plaque that forms on enamel surfaces over the lifetime of the animal. The soft dental plaque upon tooth surfaces is comprised of bacteria and fluids that over time harden into a crust, called calculus, as calcium phosphate deposits into plaque layers on the enamel (Kinaston et al., 2019). Any plant microfossils that became embedded in the dental calculus are protected from further deterioration by enzymes or mastication (Henry, 2012; White, 1997). The trapped microremains can be used to reconstruct the approximate diet of an extinct organism, with the most diagnostic recoverable microfossils including phytoliths (siliceous bodies within plant cells), pollen grains (male plant reproductive gametes), and starch grains (energy storage bodies within plant cells) (Henry, 2012). Further, due to the distinct morphological characteristics of plant microfossils, identification of specific taxa is possible even to the species level in certain cases (Henry, 2012; Piperno, 2006). Regarding the *Merychippus* species, utilization of plant microremains can thus allow for direct identification of dietary plant taxa.

The objective of this study was to use plant microfossils from *Merychippus* sp. calculus samples collected from four fossil dig sites of Barstovian age (15.9-12.5 MYA), to 1) quantify and compare dietary constituents for *Merychippus* sp., and 2) statistically compare *Merychippus* calculus samples from the four different dig sites. More broadly, this study infers aspects of the paleo-environment of *Merychippus* sp. based on plant evidence and serves as a test of using dental calculus as a medium for reliable dietary evaluation in extinct herbivores.

Methodology

Four *Merychippus* sp. dental calculus samples were obtained from the Smithsonian National Museum of Natural History collections and processed to procure microfossils. Each calculus sample was collected from a single isolated tooth that came from one of four different sample sites: (1) Group A: Norden Bridge Quarry, Valentine Formation, Brown County, Nebraska (Miocene: Barstovian age) (Fig. 2a), (2) Group B: Dawes County, Nebraska (Miocene: Barstovian age) (Fig. 2b), (3) Group C: Crooked River, Mascall Formation, Crook County, Oregon (Miocene: Barstovian age) (Fig. 2c), (4) Group D: Chesapeake beach, Calvert Formation, Maryland (Miocene: Barstovian age) (Fig. 2d). The Valentine Formation is 53-69 m thick and features the major sedimentary constituents of clastic sandstone, carbonate limestone, and clastic siltstone (United States Geologic Survey, n.d.-b). The Mascall Formation has a minimum thickness of 353 m with the major constituent of felsic igneous rhyolite, originating from viscous lava flows (Bestland et al., 2008; United States Geologic Survey, n.d.-c). Finally, the Calvert Formation is 0-46 m thick and contains the major constituents of coarse-detrital unconsolidated sand, and fine-detrital unconsolidated clay (United States Geologic Survey, n.d.-a).

A limited sample size of four fossils was utilized due to the considerable effort that was eventually required to quantify the microscopic remains in these calculus samples, and because a similar number (5) of dental specimens were utilized in previous paleodiet studies as an appropriate number to effectively determine components of a paleodiet (Henry and Piperno, 2008). Further, there is currently no precedent for a determinate number of microfossils needed to accurately characterize paleodiet, but studies utilizing microfossils as a sole means of

paleodiet determination have used between 30-201 microfossils (Ciochon et al., 1990; Gobetz and Bozarth, 2001; Henry and Piperno, 2008).

Recovery of Calculus from Fossil Specimens

Each separate *Merychippus* sp. fossil tooth specimen was scraped with a dental curette to collect as much calculus as possible without damaging the fossil. Following removal of the plaque, the collected material was stored in 14-ml glass collection tubes prior to microfossil isolation. The curette was wiped with a 70% ethanol solution, as required within the Smithsonian National Museum of Natural History collections facilities, between individual specimens to prevent cross-contamination.

Laboratory Processing of Calculus Samples

To initiate the isolation of microfossils from each calculus sample, the microfossils needed to be separated from the layers of calcified plaque binding them and preventing identification. The isolation methods utilized in this study were adapted from Henry and Piperno (2008). The isolation of microfossils began with manual crushing of the calculus to a powder with a dental curette, rinsing the curette with deionized water between individual samples to prevent cross-contamination. Once powdered, the samples were deflocculated by funneling 0.1 g of each sample into centrifuge tubes with enough solution of 10% sodium hexametaphosphate to completely submerge the calculus, and leaving samples for 24 hours in a fume hood. Afterward, the samples were sonicated for five minutes and centrifuged at 2000 rpm for two minutes, the supernatant was pipetted off, and the samples were rinsed twice with deionized water. Next, a 10% solution of HCl was added to the samples and samples were left in the fume hood for another 12 hours. Finally, the samples were rinsed twice with deionized water.

Two microscopic slides were prepared from each calculus sample. Slides were made by first pipetting 900 µl of mineral oil into each centrifuge tube of processed calculus, and then vigorously stirring the solution with a dental curette. Subsequently, 60 µl of sample were collected from approximately 5 cm below the meniscus of the mineral oil within each tube while the calculus was still settling, to ensure that larger flakes of calculus would not block the pipette. The pipetted material was then placed onto the slide, covered with a coverslip, and sealed with clear nail polish. The dental curette was rinsed between samples with deionized water to prevent cross-contamination.

Data Collection and Imaging of Samples

The total number of microfossils on each slide was recorded using a light compound microscope (typically at 200x or 400x magnification), and each microfossil was initially identified as belonging to one of four broad categories: vessel element, tracheid, phytolith, or unidentifiable plant fiber.

Vessel elements are indicative of microfossils that originated from vascular tissue of hardwood trees such as oak or elm. Tracheids are another type of vascular tissue, and act as indicators for microfossils from either softwood trees such as pine or hardwood trees (Butterfield and Meylan, 1980; Ilvessalo-Pfäffli, 1995). In order to differentiate the source of a tracheid as originating from a softwood or hardwood tree, the morphology of bordered pit pairs within the tracheids was used as a diagnostic tool (Fig. 3). More specifically, presence or absence of bordered pits within a tracheid was used to determine source, as the absence of this feature is indicative of tracheids originating within hardwoods. Likewise, presence of bordered pits is associated with tracheids originating from softwoods (Ilvessalo-Pfäffli, 1995). Phytoliths are the silicified (opalline) remnants of cells from various plant species, and are particularly useful to

distinguish between types of grasses, which have particularly high silica content (Henry, 2012). Lastly, unidentifiable plant fibers were classified, for the purposes of this study, as any organic plant material found in the slides that could not be identified to any other category but still represented plant matter that was consumed by *Merychippus*.

Vessel elements, tracheids, and phytoliths were identified using a morphological handbook that allows for finer taxonomic distinction between the three types of microfossils, such as genus level (Ilvessalo-Pfäffli, 1995). All microfossils were imaged using a Leica DM6b microscope and Zeiss Axioscope.A1 microscope in the Microscopy Suite in the Biology Department at James Madison University.

Results

A total of 70 microfossils were identified within the Group A assemblage (USNM 352521) (Valentine Fmn, NE), 131 microfossils were identified within the Group B assemblage (USNM 413190) (Dawes Co, NE), 99 microfossils were identified within the Group C assemblage (USNM 20088) (Mascall Fmn, OR), and 93 microfossils were identified within the Group D assemblage (USNM 215140) (Calvert Fmn, MD) (Table 1; Table 2, Table 3: Table 4). For the purpose of this study, microfossils were categorized conservatively as either "definite" or "possible." Microfossils denoted as definite were identified confidently within their respective categories, and any microfossil that could not be identified with full confidence was labelled as a possible constituent of the respective sample group. However, additional clarification is needed regarding microfossils categorized as "possible sedge phytoliths" beyond what is implied by the label possible or definite. The phytoliths labelled as possible sedge (C_4) have a morphology that does not entirely rule out the possibility that these phytoliths are derived from C_3 grass species. For instance, phytoliths from wheat-type (C_3) grasses have a similar morphology (conical with scalloped margin) to those from sedges.

Group A contained the least number of microfossils (70) of the four groups and the least amount of diversity among the microfossils identified (Table 1; Fig. 4a). Within Group A, definite and possible hardwood microfossils made up the largest relative percentages of all categories, representing 53% and 24% of the group, respectively (Fig. 4a). Among the hardwood microfossils, a prevalent type was definite single plate phytoliths (36) from leaf epidermal plate aggregates (Table 1, Fig. 5a). All microfossils within the Group A assemblage were recovered from a single *Merychippus* sp. tooth originating from the Valentine Formation in Brown County, Nebraska.

Group B contained the most microfossils (131) of the four groups, with definite hardwood microfossils, definite pooid phytoliths, and possible grass microfossils comprising 20%, 20%, and 17% of the group, respectively (Table 2; Fig. 4b). The most common specific types of microfossils present within Group B were definite plant fibers (20), definite trapezoidal pooid phytoliths (19), and definite single plate phytoliths (14) from leaf epidermal plate aggregates (Table 2, Fig. 6b, 6d, 6e). All microfossils within the Group B assemblage were recovered from a single *Merychippus* sp. tooth found in Dawes County, Nebraska.

Similar to Group A, definite and possible hardwood microfossils were the most prevalent types identified in Group C, comprising 22% and 17% of total microfossils identified (Fig. 4c). Group C also featured a relatively large percentage (15%) of definite pooid grass phytoliths in addition to the hardwood microfossils (Fig. 4c, Fig. 7g). A total of 99 microfossils was identified from Group C, with the most common specific microfossils being definite (12) and possible (11) single plate phytoliths from leaf epidermal aggregates (Table 3, Fig. 7b). All microfossils within the Group C assemblage were recovered from an isolated single *Merychippus* sp. tooth originating from the Mascall Formation in Crook County, Oregon.

Within Group D, which contained 93 total identified microfossils, the dominant categories were definite hardwoods (23%), definite tracheids (16%), and possible sedge phytoliths (16%) (Table 4; Fig. 4d). The largest groups of specific microfossils were possible sedge phytoliths (15), and definite (11) and possible (10) single plate phytoliths from leaf epidermal aggregates (Table 4; Fig. 8h, 8f). All microfossils within the Group D assemblage were recovered from a single *Merychippus* sp. tooth recovered from the Calvert Formation in Calvert County, Maryland.

Each microfossil assemblage group was compared to every other assemblage group using chi-squared tests, as a preliminary determination of whether the observed distribution of microfossils from each tooth was statistically different. The chi-square tests determined that each assemblage of microfossils from each *Merychippus* sp. tooth specimen was significantly different from every other assemblage, as the two-tailed p-value in every comparison was less than 0.0001. Chi-squared tests were performed using an online calculator (GraphPad, 2018).

Discussion

Microfossil assemblages were recovered from four individual isolated *Merychippus* sp. teeth from regionally and geologically different locations within the United States. Group A contained microfossils recovered from a single *Merychippus* sp. tooth originating from the Valentine Formation in Brown County, Nebraska. Within the Group A microfossil assemblage, there were definite and possible single plate phytoliths from leaf epidermal plate aggregates, pooid grass phytoliths, vessel elements, possible grass phytoliths and definite plant fibers (Table 5; Fig. 4a; Fig. 5). Based on the morphology of articulated leaf epidermal plate phytoliths, as well as the presence of vessel elements, there is indication that foliage from hardwood trees was included in the diet of this individual *Merychippus* sp., specimen USNM 352521 (Table 1; Fig. 5a) (Bozarth, 1992; Geis, 1973; Ilvessalo-Pfäffli, 1995; Rovner, 1971).

The phytoliths identified in Group A included pooid phytoliths derived from C_3 grasses within Subfamily Pooidae, Family Pooaceae, and possible phytoliths, which may be the remnants of nonspecific grass species that undergo a C_3 or C_4 photosynthetic pathway (Table 1; Fig. 5c) (Brown, 1984; Twiss, 1992; Twiss et al., 1969). Further, the definite plant fibers in this first assemblage were not indicative of any specific category of plant that could have been consumed by *Merychippus*, but were included as remnants of plant derived tissue (Table 1; Fig. 5b) (Ilvessalo-Pfäffli, 1995). The predominance of definite hardwood microfossils, the inclusion of definite pooid phytoliths, and the presence of possible grass phytoliths, suggest that *Merychippus* sp. from the Valentine Formation had a potentially mixed browse/grass diet that included grasses that underwent C_3 photosynthesis and possibly C_4 photosynthesis.

The Group B microfossil assemblage was recovered from a single *Merychippus* sp. tooth found in Dawes County, Nebraska. Within this assemblage, there was a wide array of microfossils that include definite and possible tracheids, sedge-type phytoliths, pooid phytoliths, unspecified grass phytoliths, plant fibers, single plate phytoliths from leaf epidermal plate aggregates, and vessel elements (Table 5; Fig. 4b; Fig. 6). Similar to Group A, the morphology of the leaf epidermal plate phytoliths and vessel elements are consistent with tissue derived from hardwood trees (Table 2; Fig. 6e) (Bozarth, 1992; Geis, 1973; Ilvessalo-Pfäffli, 1995; Rovner, 1971). Within a diverse array of grass phytoliths, there was a predominance of phytoliths from C_3 pooid grasses and from indefinite grass species that underwent either C_3 or C_4 photosynthesis (Table 2; Fig. 4b; Fig. 6d, 6c) (Brown, 1984; Twiss, 1992; Twiss et al., 1969). The remaining phytoliths of the assemblage featured morphological characteristics consistent with sedges (Family Cyperaceae), which are a grass-like C_3/C_4 photosynthetic group that contains species that grow in a variety of wetland and forested conditions (Table 2; Fig. 4b; Fig. 6f) (Hesla et al., 1982; Ollendorf, 1992; Waterway et al., 2009).

Tracheid microfossils in the assemblage were identified but not characterized further as definitively derived from softwood or hardwood species (Table 2; Fig. 6a). Instead, the presence of tracheids was interpreted as an indicator for the likely incorporation of softwood plant material into a diet, as tracheids are vascular cells that comprise the majority of the softwood axial system (Butterfield and Meylan, 1980; Ilvessalo-Pfäffli, 1995). The microfossils labelled as definite or possible plant fibers included any microfossil that could not be identified as anything other than a material that was plant-derived or potentially plant-derived, respectively (Table 2; Fig. 6b) (Ilvessalo-Pfäffli, 1995). Within Group B, the inclusion of a wide array of definite microfossils that incorporated sedge phytoliths, phytoliths of C_3 and C_4 grasses, and microfossils

derived from hardwood and possibly softwood trees, suggest a potential diet consistent with a mixed browser/grass feeder for *Merychippus* sp. around the Nebraska county from which the tooth was recovered.

The Group C microfossil assemblage was recovered from an isolated single *Merychippus* sp. tooth originating from the Mascall Formation in Crook County, Oregon. Comparable to the previous microfossil assemblage of Group B, the Group C assemblage was diverse, with the inclusion of possible and definite tracheids, sedge phytoliths, pooid grass phytoliths, leaf epidermal plate aggregates, vessel elements, and definite plant fibers (Table 5; Fig. 4c; Fig. 7). The morphology of single plate phytoliths from leaf epidermal plate aggregates and vessel elements within the assemblage bear resemblance to tissue originating from hardwood trees (Table 3; Fig. 7b) (Bozarth, 1992; Geis, 1973; Ilvessalo-Pfäffli, 1995; Rovner, 1971). As for Group B, tracheid microfossils in this assemblage were not characterized definitively as originating from hardwoods or softwoods. The tracheids within the assemblage were identified as evidence for the likely inclusion of softwood tree species into a paleodiet (Table 3; Fig. 7e) (Butterfield and Meylan, 1980; Ilvessalo-Pfäffli, 1995). Among the definite phytoliths of the assemblage, C_3 pooid grass phytoliths were most prevalent, followed by grass phytoliths of either C_3 or C_4 photosynthetic type, and C_3/C_4 sedge phytoliths belonging to the Family Cyperaceae (Fig. 4c; Fig. 7g, 7d, 7c) (Brown, 1984; Hesla et al., 1982; Ollendorf, 1992; Twiss, 1992; Twiss et al., 1969). There were no possible plant fibers present in this assemblage, and the definite plant fibers were unidentifiable to anything other than remnants of plant-derived tissue (Table 3; Fig. 7a) (Ilvessalo-Pfäffli, 1995). Within Group C, a diversity of definite microfossils that include sedge phytoliths, phytoliths of C_3 and C_4 grasses, and microfossils derived from

hardwood and possibly softwood trees, suggest a potential mixed browse/grass feeding preference for *Merychippus* sp. from the Mascall Formation.

However, unique to Group C were the incidence of freshwater sponge spicules and shards of volcanic glass. Despite not being plant-derived, these microscopic particles provide information regarding the paleoenvironment of *Merychippus* sp. native to the area encompassed by the Mascall Formation (Table 3; Fig. 7h, 7f). Presence of freshwater sponge spicules in dental calculus could indicate a wet paleoenvironment that may have included bodies of freshwater in which the sponges would have thrived (Manconi and Pronzato, 2014; Pisera et al., 2019). Further, the inclusion of sponge spicules within the assemblage does not suggest deliberate consumption of sponges, rather spicules may have become incorporated within dental calculus as an environmental contaminant of drinking freshwater (Warinner et al., 2015). The volcanic rock shards were morphologically identified as tephra (Fig. 7f) (Swindles et al., 2010), and are likely soil contaminants from the Mascall Formation, as the Formation includes ash-flow tuffs from volcanic activity in the area (McClaughry et al., 2009; United States Geologic Survey, n.d.-c). These exposed and weathering beds of volcanic rock could have ended up as part of a grit layer on the plants consumed by local herbivores in this part of ancient Oregon.

The Group D microfossil assemblage was recovered from a single *Merychippus* sp. tooth recovered from the Calvert Formation in Calvert County, Maryland. Within this assemblage, microfossils included definite and possible tracheids, sedge phytoliths, pooid grass phytoliths, grass phytoliths, hackberry seed cells, leaf epidermal plate aggregates, vessel elements, and definite plant fibers (Table 5; Fig. 4d; Fig. 8). As with other groups, single plate phytoliths from leaf epidermal plate aggregates and vessel elements are characteristic of hardwood tree tissue (Table 4; Fig. 8f) (Bozarth, 1992; Geis, 1973; Ilvessalo-Pfäffli, 1995; Rovner, 1971). Consistent

with the classification of tracheids in Groups B and C, the tracheids identified from Group D suggest the likely inclusion of softwood tree tissue in the diet of the *Merychippus* sp. specimen USNM 215140 (Table 4; Fig. 8e) (Butterfield and Meylan, 1980; Ilvessalo-Pfäffli, 1995).

The pattern of relative definite phytolith frequencies in the Group D assemblage mirrors that of Group C. Phytoliths originating from C_3 pooid grasses were the most common, grass phytoliths derived from C_3 or C_4 grasses were the second most common, and C_3/C_4 sedge phytoliths identified to Family Cyperaceae were the least common among the definite phytolith categorizations (Fig. 4d; Fig. 8c, 8d, 8a) (Brown, 1984; Hesla et al., 1982; Ollendorf, 1992; Twiss, 1992; Twiss et al., 1969). However, there was a prevalence of possible sedge phytoliths in Group D. Due to the indefinite morphology of these phytoliths, they likely indicate the consumption of Cyperaceae species, and more general consumption of grasses of either C_3 or C_4 photosynthetic type (Brown, 1984; Ollendorf, 1992; Twiss et al., 1969). Of the possible sedge phytoliths included in this category, one phytolith featured morphology that more specifically indicated that it could potentially be derived from a species of wheat-type grass, if not truly a sedge phytolith (Fig. 8h) (Ball et al., 2009). Additionally, among the phytoliths identified as possibly derived from C_3 or C_4 grasses, a single phytolith featured morphology that indicated that it was potentially a fragment of a grass inflorescence (bud) (Table 4) (Novello and Barboni, 2015; Rajendiran et al., 2012). The presence of this microfossil may suggest that grass inflorescences, along with grass leaves, were masticated as *Merychippus* fed on grasses, which is true of most herbivores and is a further endorsement of dental plaque as a reliable dietary indicator.

Another distinct facet of the Group D microfossil assemblage was the isolation of definite and possible seed cells from hackberry-type plants that suggest the incorporation of *Celtis* sp. or

similar seeds into the calculus on the tooth (Table 4; Fig. 8b) (Bozarth, 1987; Gobetz and Bozarth, 2001). Possible plant fibers were not present in this assemblage, and the definite plant fibers of the assemblage were only indicative of plant-derived tissue (Table 4; Fig. 8g) (Ilvessalo-Pfäffli, 1995). Within Group D, the inclusion of sedge phytoliths, cells of hackberry seed, phytoliths of C_3 and C_4 grasses, and microfossils derived from hardwood and possibly softwood trees, again suggest a potential mixed browse/grass diet for *Merychippus* sp. from the Maryland region Calvert Formation.

Regarding the paleoenvironment of *Merychippus*, the early Miocene is presumed to be a time period during which grasslands were beginning to expand in North America, and the grass species that dominated the ecosystem were most likely C_3 grasses (Macfadden, 2000; Wang et al., 1994). Previous studies on the paleodiet of *Merychippus* have indicated that equids of this genus were a mixed feeding type incorporating both browse and grasses, and a high prevalence of C_3 plants in general (Hayek et al., 1992; Pérez-Crespo et al., 2016; Wang et al., 1994). In the present study, microfossils identified from the four assemblages provide further evidence that *Merychippus* in North America consumed a mixed browse/grass diet, and present within each assemblage were C_3 plants. The findings of this study corroborate previous research with regard to the mixed browse/grass feeding type and the inclusion of C_3 plants in the diet of *Merychippus*. Moreover, within each assemblage were definite pooid phytoliths which support an expected composition of North American Miocene ecosystems of grasslands dominated by C_3 grass species prevailing in moist, temperate habitats (Macfadden, 2000; Wang et al., 1994).

Conclusion

Regarding the objectives of this study, calculus from four *Merychippus* isolated individual teeth yielded diagnostic microfossils that were utilized to quantify dietary constituents of *Merychippus* and generalize aspects of the paleoenvironment of *Merychippus*. Overall, the results of this study demonstrate that microfossils can be successfully isolated using the relatively new method of dental calculus analysis, and can be identified to a level of certainty that is consistent with previous findings. Microfossil assemblages from Groups A, B, C, and D all featured definite phytoliths from fragmented leaf epidermal plate aggregates, as well as vessel element microfossils, indicating that hardwood plant species comprised a component of paleodiet in each case (Table 1; Table 2, Table 3: Table 4) (Bozarth, 1992; Geis, 1973; Ilvessalo-Pfäffli, 1995; Rovner, 1971). Groups B, C, and D featured definite sedge microfossils and all groups contained definite pooid phytoliths that were identified as derived from sedges from Family Cyperaceae and grasses from the Poaceae Subfamily Pooideae, respectively (Table 1-4) (Ollendorf, 1992; Twiss, 1992). Tracheid microfossils were isolated in all groups except Group A and were interpreted as an indication for inclusion of softwood plant species. Definite plant fibers present in all groups were inferred as general indicators of consumption of plant tissue (Table 1-4) (Butterfield and Meylan, 1980; Ilvessalo-Pfäffli, 1995). Each microfossil assemblage group differed significantly from every other assemblage.

Concerning the paleoenvironment of *Merychippus*, a predominance of hardwood microfossils in all groups suggests that hardwood tree species may have been shared dietary components (Fig. 4). Additionally, the presence of pooid grass phytoliths in all assemblages indicates the inclusion of grasses of C_3 character in the early Miocene environment of *Merychippus*, which is consistent with the presumed paleoenvironmental composition of C_3

dominated grasslands of North America during the same period (Twiss, 1992; Wang et al., 1994).

Paleodiet studies of extinct equids that solely utilize plant microfossils in dental calculus are limited. Although this study was constrained to only four *Merychippus* tooth specimens, there were a profusion of microfossils isolated from the calculus samples collected which allowed for analyses to be made concerning the paleodiet and paleoenvironment of *Merychippus* within the early Miocene of North America. Further paleodiet studies of this type may provide greater clarity towards the paleoenvironment and paleodiet of the modern equid ancestors.

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Appendix: Figures and Tables

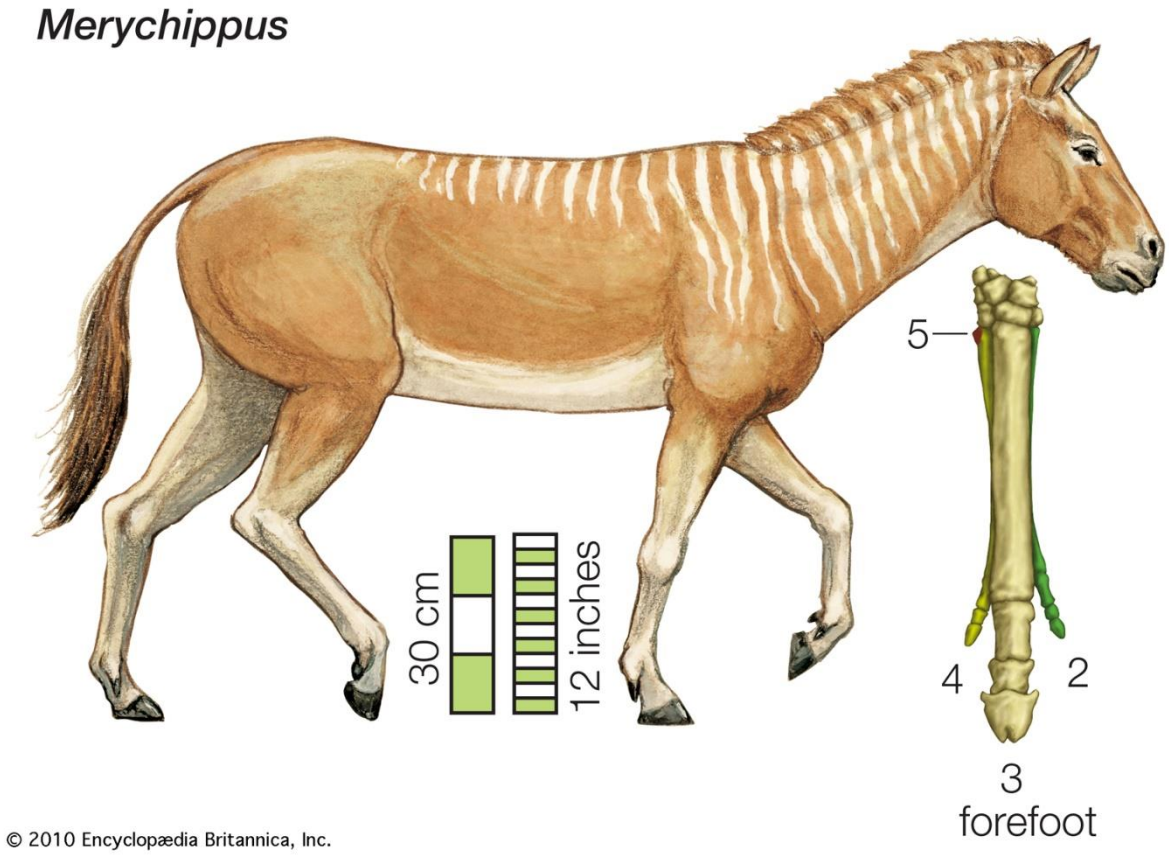

Fig. 1. Illustration of extinct tridactyl equid *Merychippus* sp. Photo excerpted from The Editors of *Encyclopedia Britannica* (2017).

Fig. 2. Photos of *Merychippus* tooth specimens. (a) *Merychippus insignis* molar (USNM 352521) with quarter for scale; (b) *Merychippus* sp. molar (USNM 413190) with half-dollar for scale; (c) *Merychippus* sp. molar (USNM 20088) with quarter for scale; (d) *Merychippus* sp. molar (USNM 215140) with 15 cm bar.

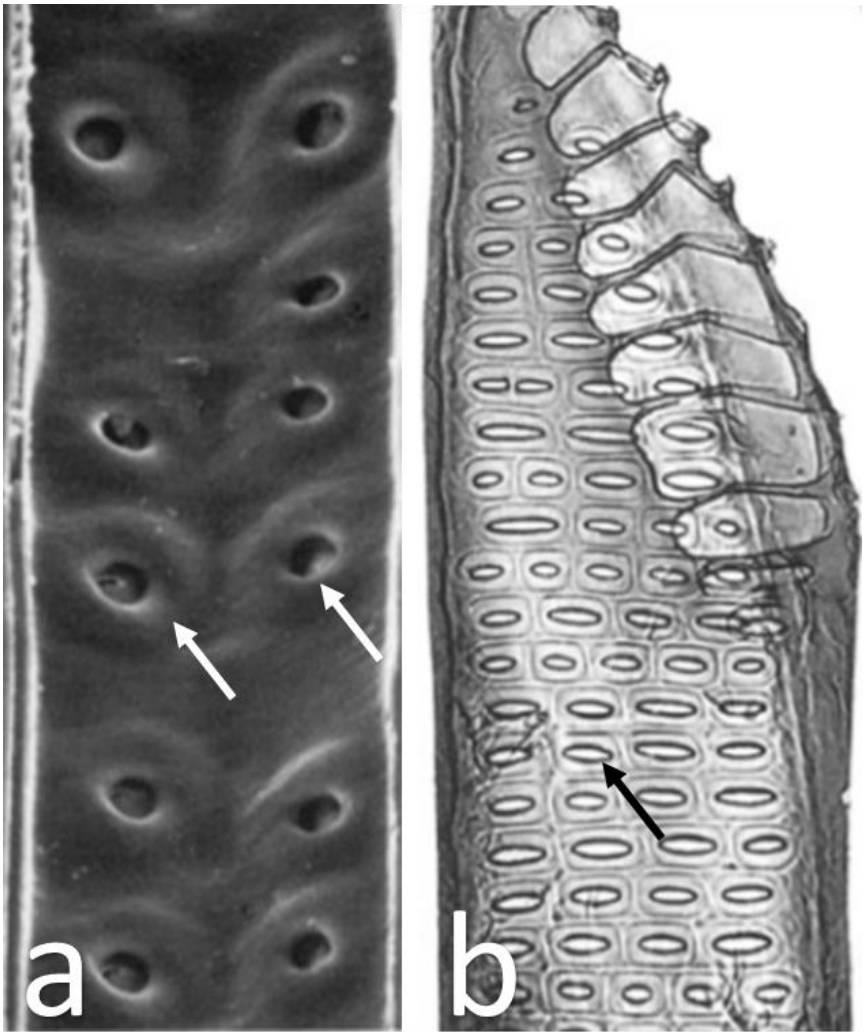

Fig. 3. Examples of pit morphology. (a) Example of a bordered pit pair within a softwood tracheid (*Taxodium distichum*). Photo modified and excerpted from (Butterfield and Meylan, 1980). (b) Example of pitting within a hardwood vessel element (*Liriodendron tulipifera*). Photo modified and excerpted from (Ilvessalo-Pfäffli, 1995).

Table 1.

Frequencies of microfossils in Group A (Valentine Fmn, NE) isolated from USNM 352521.

Group A microfossils	**Number of data points**
Definite	
Hardwood microfossils	
Leaf epidermal plate aggregate	36
Vessel element	1
Pooid grass phytolith microfossils	
Pooid trapezoidal phytolith	1
Pooid sinuous short cell	1
Plant fiber microfossils	
Plant fiber	7
Possible	
Hardwood microfossils	
Leaf epidermal plate aggregate	16
Vessel element	1
Pooid grass phytolith microfossils	
Pooid sinuous short cell	2
Grass phytolith microfossils	
Phytolith	4
Bulliform phytolith	1
Total microfossils	70

Table 2.

Frequencies of microfossils in Group B (Dawes Co, NE) isolated from USNM 413190.

Group B microfossils	**Number of data points**
Definite	
Hardwood microfossils	
Leaf epidermal plate aggregate	14
Vessel element	6
Vessel element fragment	6
Tracheid microfossils	
Tracheid	3
Pooid grass phytolith microfossils	
Pooid short cell	1
Pooid sinuous short cell	6
Pooid trapezoidal phytolith	19
Grass phytolith microfossils	
Short cell	5
Spiny long cell	1
Spiny phytolith	2
Bulliform phytolith	1
Long cells	4
Ovoid phytolith	1
Sedge phytolith microfossils	
Sedge phytolith	2
Plant fiber microfossils	
Plant fiber	20
Possible	
Hardwood microfossils	
Leaf epidermal plate aggregate	6
Vessel element	1
Vessel element fragment	1
Tracheid microfossils	
Tracheid fragment	2
Pooid grass phytolith microfossils	
Pooid short cell	4
Pooid trapezoidal phytolith	1
Grass phytolith microfossils	
Short cell	6
Blocky phytolith	9
Bulliform phytolith	1
Phytolith	6
Sedge phytolith microfossils	
Sedge phytolith	2
Plant fiber microfossils	
Plant fiber	1
Total microfossils	131

Table 3.

Frequencies of microfossils in Group C (Mascall Fmn, OR) isolated from USNM 20088.

Group C microfossils	**Number of data points**
Definite	
Hardwood microfossils	
Leaf epidermal plate aggregate	12
Vessel element	6
Vessel element fragment	4
Tracheid microfossils	
Tracheid	6
Pooid grass phytolith microfossils	
Pooid trapezoidal phytolith	7
Pooid short cell	3
Pooid sinuous short cell	5
Grass phytolith microfossils	
Long cell	2
Blocky short cell	1
Saddle shaped short cell	1
Short cell	1
Sedge phytolith microfossils	
Sedge phytolith	2
Plant fiber microfossils	
Plant fiber	8
Sponge spicule microfossils	
Sponge spicule	8
Tephra	
Tephra fragment	2
Possible	
Hardwood microfossils	
Leaf epidermal plate aggregate	11
Vessel element fragment	6
Tracheid microfossils	
Tracheid fragment	3
Tracheid tip	2
Pooid grass phytolith microfossils	
Pooid trapezoidal phytolith	4
Pooid sinuous short cell	1
Grass phytolith microfossils	
Short cell	2
Long cell	1
Sedge phytolith microfossils	
Sedge phytolith	1
Total microfossils	99

Table 4.

Frequencies of microfossils in Group D (Calvert Fmn, MD) isolated from USNM 215140.

Group D microfossils	**Number of data points**
Definite	
Hardwood microfossils	
Leaf epidermal plate aggregate	11
Vessel element	3
Vessel element with intervessel pitting	7
Tracheid microfossils	
Tracheid	6
Tracheid fragment	9
Pooid grass phytolith microfossils	
Pooid sinuous short cell	1
Pooid trapezoidal phytolith	7
Grass phytolith microfossils	
Short cell	1
Spiny long cell	1
Long cell	1
Bulliform phytolith	2
Sedge phytolith microfossils	
Sedge short cell	1
Plant fiber microfossils	
Plant fiber	1
Hackberry microfossils	
Hackberry seed cell	2
Possible	
Hardwood microfossils	
Leaf epidermal plate aggregate	10
Vessel element	1
Tracheid microfossils	
Tracheid tip	1
Pooid grass phytolith microfossils	
Pooid sinuous short cell	2
Grass phytolith microfossils	
Long cell	1
Phytolith	2
Short cell	6
Grass inflorescence fragment	1
Sedge phytolith microfossils	
Sedge/spiny phytolith	13
Sedge/spiny-domed phytolith	1
Sedge/wheat phytolith	1
Hackberry microfossils	
Hackberry seed cell	1
Total microfossils	93

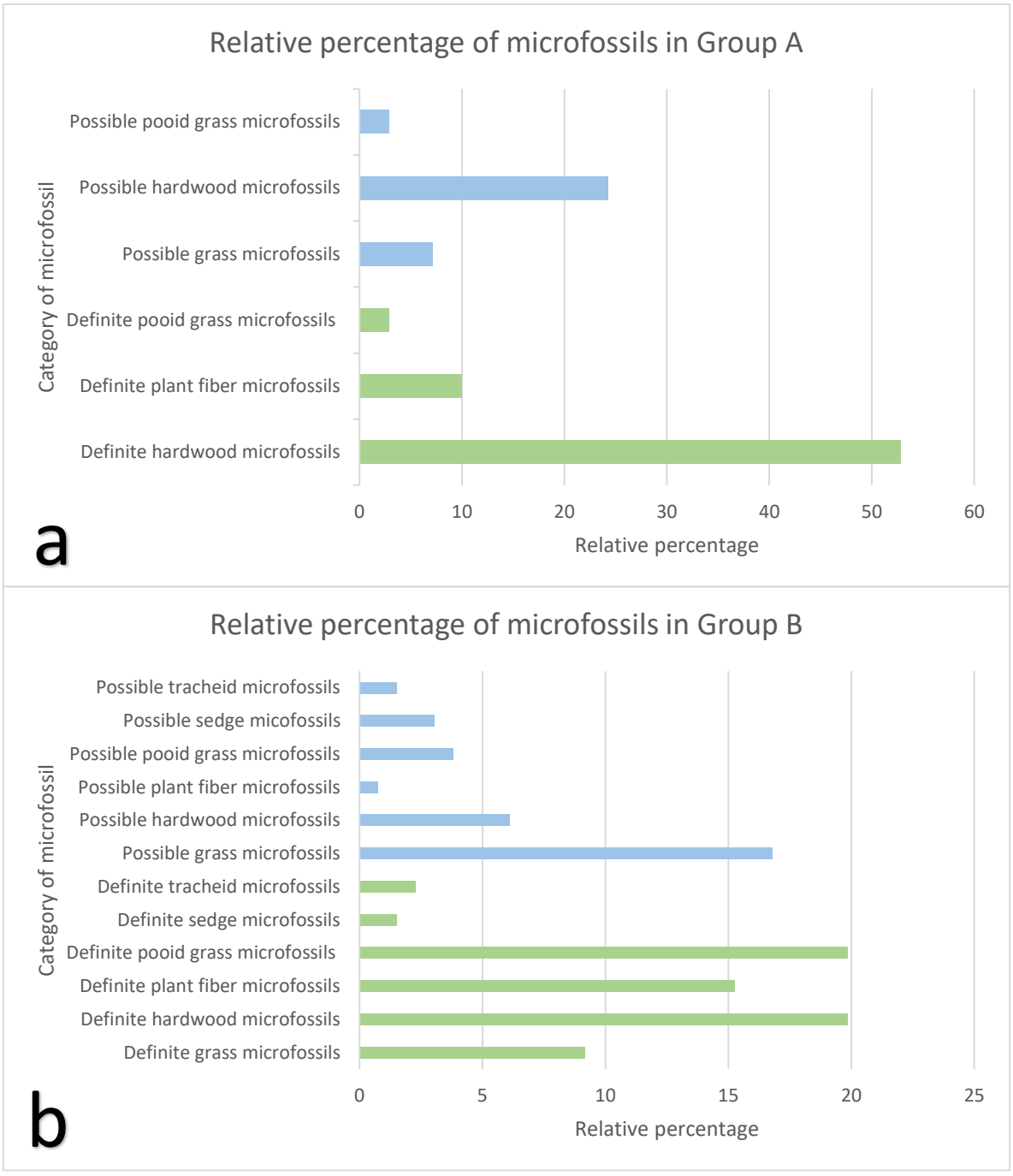

Fig. 4. Graph of the relative percentage of microfossils within Groups A-D. (a) Relative percentage of microfossils within Group A (Valentine Fmn, NE) derived from microfossil categorizations in Table 1; (b) Relative percentage of microfossils within Group B (Dawes Co, NE) derived from microfossil categorizations in Table 2; (c) relative percentage of microfossils within Group C (Mascall Fmn, OR) derived from microfossil categorizations in Table 3; (d) relative percentage of microfossils within Group D (Calvert Fmn, MD) derived from microfossil categorizations in Table 4. Definite and possible microfossils are represented in Groups A-D by the colors, green and blue, respectively.

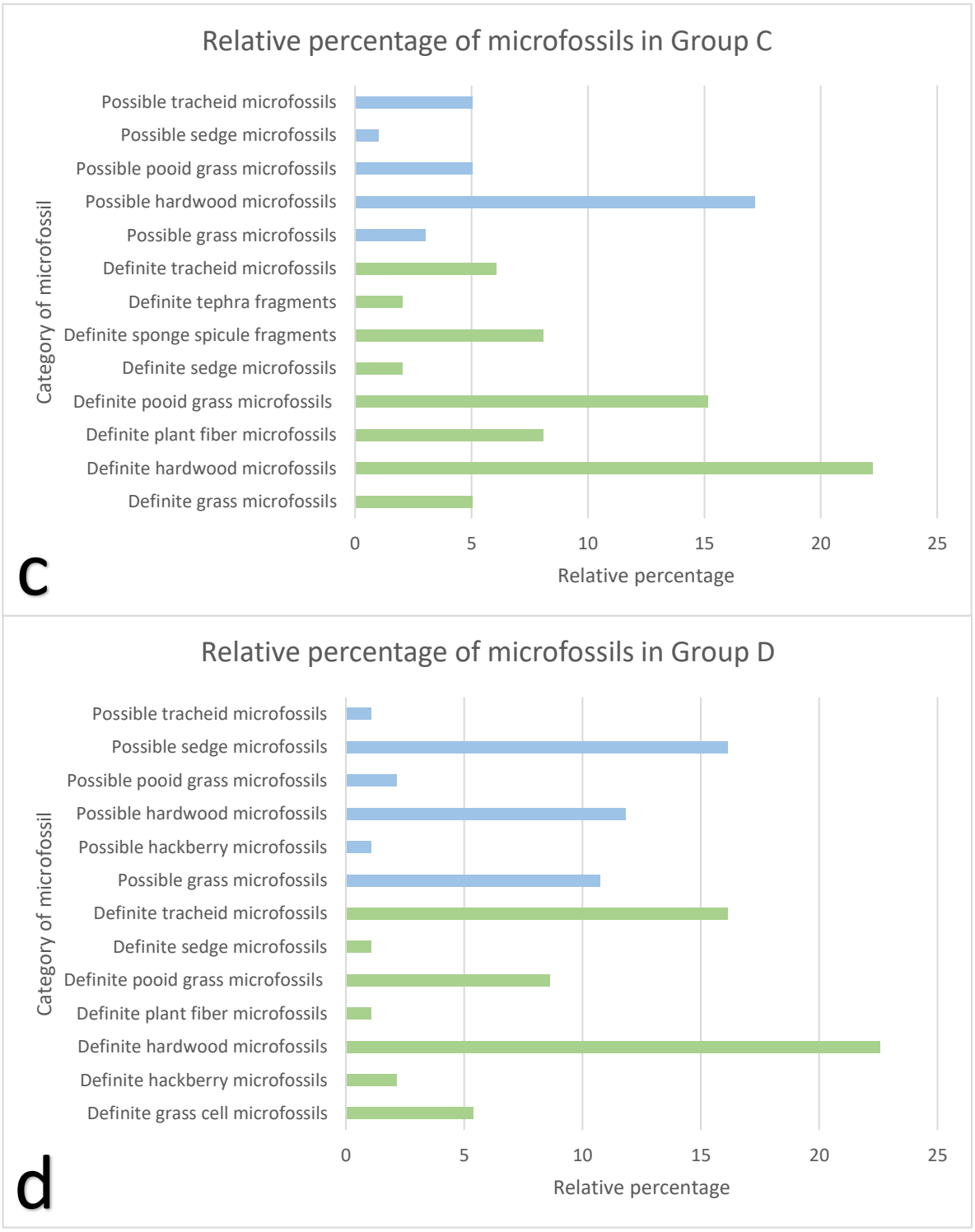

Fig. 4 (cont). Graph of the relative percentage of microfossils within Groups A-D.

Table 5. Summary of plant microfossil frequencies within diagnostic categories of Groups A-D.

Group A microfossils	**Number of data points**
Definite	
Leaf epidermal plate aggregate microfossils	36
Vessel element microfossils	1
Pooid grass phytolith microfossils	2
Plant fiber microfossils	7
Possible	
Leaf epidermal plate aggregate microfossils	16
Vessel element microfossils	1
Pooid grass phytolith microfossils	2
Grass phytolith microfossils	5
Group B microfossils	
Definite	
Leaf epidermal plate aggregate microfossils	14
Vessel element microfossils	12
Tracheid microfossils	3
Pooid grass phytolith microfossils	26
Grass phytolith microfossils	14
Sedge phytolith microfossils	2
Plant fiber microfossils	20
Possible	
Leaf epidermal plate aggregate microfossils	6
Vessel element microfossils	2
Tracheid microfossils	2
Pooid grass phytolith microfossils	5
Grass phytolith microfossils	22
Sedge phytolith microfossils	2
Plant fiber microfossils	1

Table 5 (cont). Summary of plant microfossil frequencies within diagnostic categories of Groups A-D.

Group C microfossils	**Number of data points**
Definite	
Leaf epidermal plate aggregate microfossils	12
Vessel element microfossils	10
Tracheid microfossils	6
Pooid grass phytolith microfossils	15
Grass phytolith microfossils	5
Sedge phytolith microfossils	2
Plant fiber microfossils	8
Possible	
Leaf epidermal plate aggregate microfossils	11
Vessel element microfossils	6
Tracheid microfossils	5
Pooid grass phytolith microfossils	5
Grass phytolith microfossils	3
Sedge phytolith microfossils	1
Group D microfossils	
Definite	
Leaf epidermal plate aggregate microfossils	11
Vessel element microfossils	10
Tracheid microfossils	15
Pooid grass phytolith microfossils	8
Grass phytolith microfossils	5
Sedge phytolith microfossils	1
Plant fiber microfossils	1
Hackberry microfossils	2
Possible	
Leaf epidermal plate aggregate microfossils	10
Vessel element microfossils	1
Tracheid microfossils	1
Pooid grass phytolith microfossils	2
Grass phytolith microfossils	10
Sedge phytolith microfossils	15
Hackberry microfossils	1

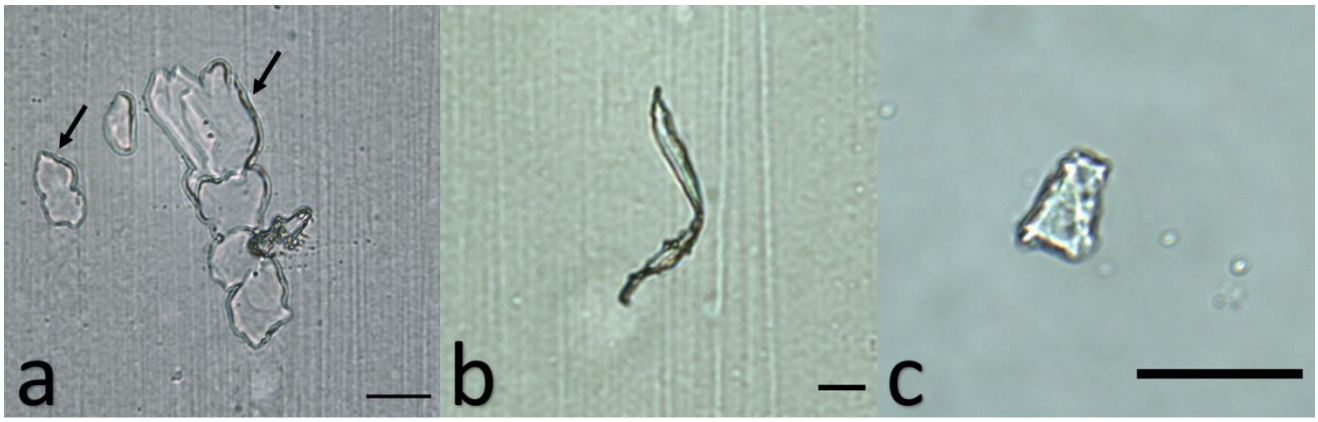

Fig. 5. Examples of definite microfossils from Assemblage A (Valentine Fmn, NE). (a) Aggregates of flat, plate-like phytoliths from leaf epidermis, surface view at 400x; (b) plant fiber, surface view at 200x; (c) trapezoidal pooid grass short cell phytolith, surface view at 400x. All microfossils were isolated from (USNM 352521); scale bars in all photographs represent 20 µm.

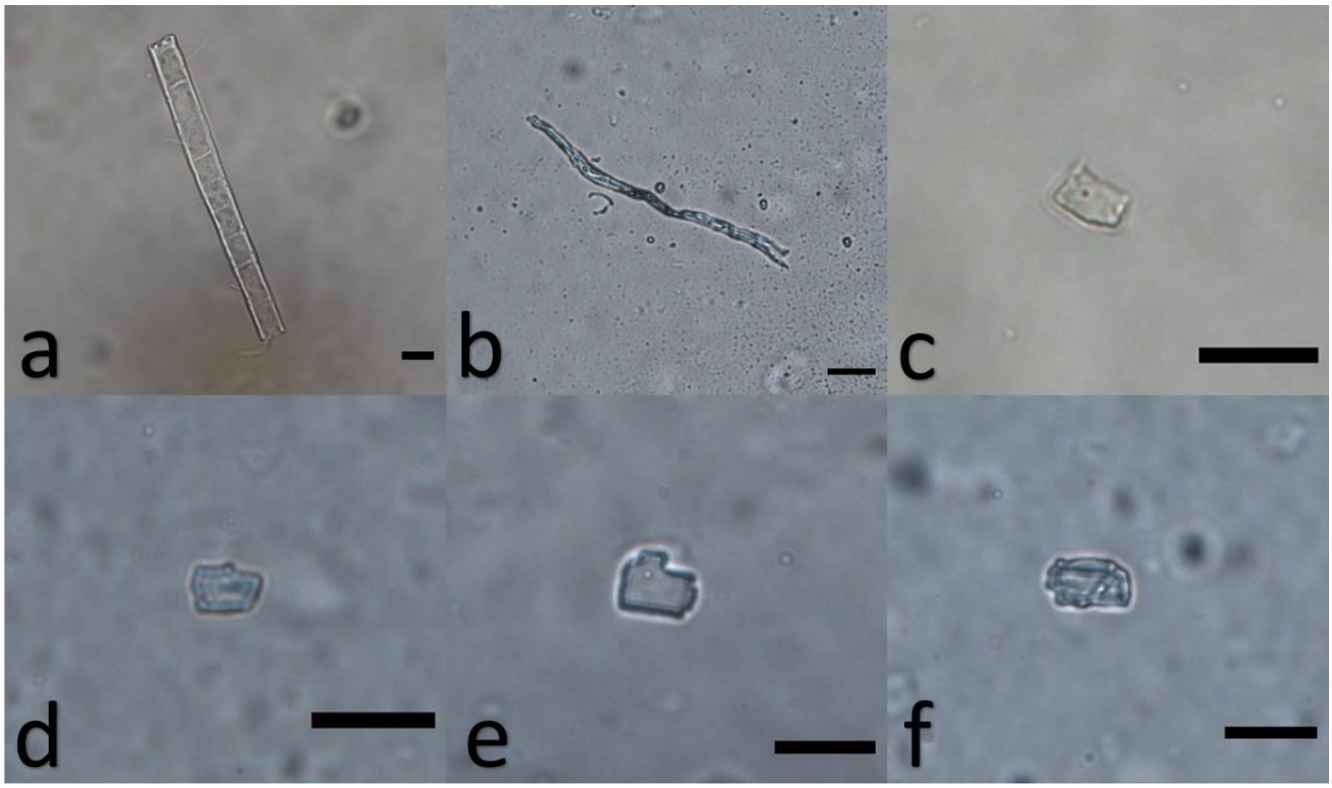

Fig. 6. Examples of definite microfossils from Assemblage B (Dawes Co, NE). (a) Tracheid, surface view at 200x; (b) indeterminate plant fiber, surface view at 200x; (c) grass short cell phytolith of indeterminate type, surface view at 400x; (d) trapezoidal short cell phytolith from pooid grass, surface view at 400x; (e) single plate phytolith from leaf epidermal plate aggregate with notched margin representing articulation points, surface view at 400x; (f) sedge-type phytolith, surface view at 400x. All microfossils were isolated from (USNM 413190); scale bars in all photographs represent 20 µm.

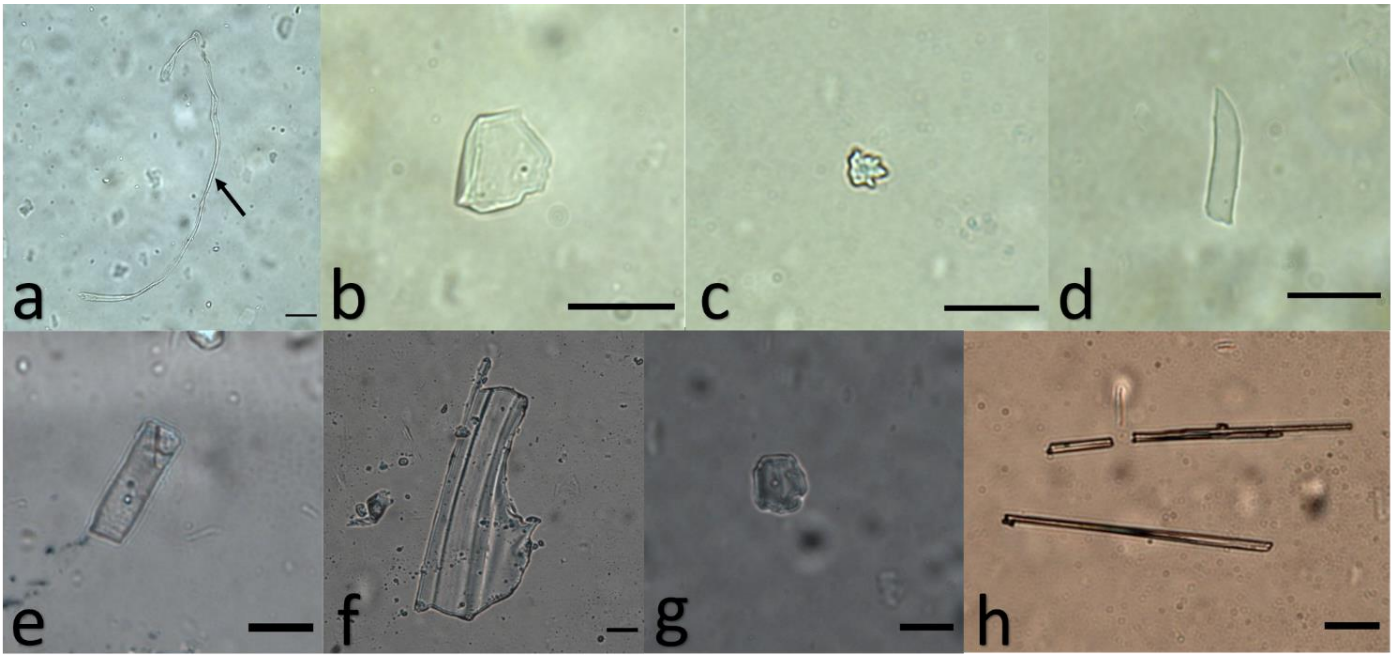

Fig. 7. Examples of definite microfossils from Assemblage C (Mascall Fmn, OR). (a) Plant fiber, surface view at 400x; (b) single plate phytolith from leaf epidermal plate aggregate with irregular margin representing articulation points, surface view at 400x; (c) spiny sedge-type phytolith, surface view at 400x; (d) grass long cell phytolith, surface view at 400x; (e) tracheid, surface view at 400x; (f) tephra fragment, surface view at 400x; (g) pooid grass sinuous short cell phytolith, surface view at 400x; (h) silica freshwater sponge spicules, surface view at 400x. All microfossils were isolated from (USNM 20088); scale bars in all photographs represent 20 µm.

Fig. 8. Examples of definite and possible microfossils from Assemblage D (Calvert Fmn, MD). (a) Conical sedge phytolith with scalloped margin, surface view at 400x; (b) possible hackberry-type seed cell phytolith, surface view at 400x; (c) pooid grass sinuous short cell phytolith, surface view at 200x; (d) grass bulliform phytolith, surface view at 200x; (e) tracheid fragment showing bordered pits, surface view at 400x; (f) single plate phytolith from leaf epidermal plate aggregate, surface view at 400x; (g) plant fiber, surface view at 400x; (h) possible sedge/wheat-type phytolith with central cone and scalloped margin, surface view at 400x. All microfossils were isolated from (USNM 215190); scale bars in all photographs represent 20 µm.